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PHENOLIC REPLACEMENTS FOR CYSTEINE IN FARNESYL TRANSFERASE INHIBITORS BASED ON CVFM

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Abstract. Compounds in which cysteine of the tetrapeptide CVFM has been replaced with a phenolic benzyl substituent inhibit farnesylation of H-ras protein by farnesyl transferase (FTase). In the most potent inhibitors (e.g., 5-chloro-2-hydroxybenzyl-VFM, IC_{50} = 0.5 μM, approx. 8 times less active than CVFM) the phenolic hydroxyl is *ortho* to the methylene linker. Inhibitory activity is influenced by substitution on the phenol ring.

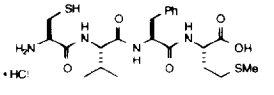
Ras is a prevalent oncogene found in several types of human cancer including colorectal carcinoma, pancreatic carcinoma, and myeloid leukemia.[1,2] Oncogenic ras mutations found in cancer cells differ from the protooncogene found in normal cells by loss of regulatory function. The proteins encoded by the ras gene family are members of a superfamily of small GTP-hydrolyzing proteins (G proteins) which must be post-translationally modified in order to be fully functional.[3-10] In the case of ras, post-translational processing is necessary to ensure that the finished protein is properly localized to the plasma membrane.[11-14]

At least three steps comprise post-translational processing of ras proteins. First, a farnesyl group is attached to the cysteine three residues from the C-terminus (at position 186 in H-ras, and at position 189 in K-ras) by reaction with farnesyl pyrophosphate, mediated by the enzyme farnesyl transferase (FTase).[15-19] Second, the carboxy-terminal three amino acids are cleaved by a specific protease.[20] Third, the carboxylic acid terminus is converted to a methyl ester by alkylation with a methyl group.[21] Farnesylation of ras proteins appears to be critical for retention of transforming ability and for various other biological indicators of ras function *in vivo*.[22]

Post-translational processing of ras proteins is signaled by a short carboxy terminus consensus sequence frequently referred to as a CAAX box, where C is cysteine, A is any aliphatic amino acid, and X is methionine, serine, or glutamine (for farnesylated proteins). Other isoprenylated small G proteins have similar sequences where X is usually leucine or occasionally phenylalanine. The CAAX sequence is believed to specify the nature of the attached isoprenyl group with the latter proteins being modified by a geranylgeranyl isoprenoid, mediated by geranylgeranyl transferase enzymes (GGTases).[23] Thus, any therapeutic agent based on FTase inhibition must contend with possible side effects arising from inhibition of GGTase(s).

Direct inhibition of FTase by short peptides[16,24-26] and peptidomimetic[27-35] substrates has been demonstrated. The tetrapeptide CVFM is a potent FTase inhibitor (IC_{50} = 0.06 μM) and is not farnesylated by the enzyme.[36]

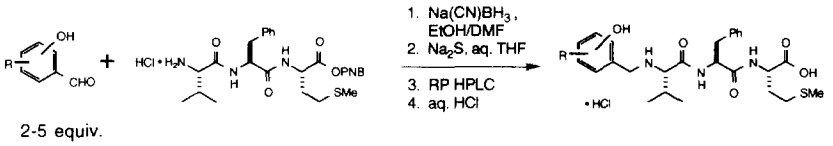
Figure 1. CysValPheMet (CVFM)



However, peptide drugs have many limitations, such as poor permeability/bioavailability and instability towards proteases. Furthermore, peptides incorporating cysteine suffer from complications due to the thiol moiety, which can undergo disulfide formation and other oxidation reactions. Hence, it would be desirable to find an FTase inhibitor which is less peptide-like and which does not contain a thiol. Unfortunately, replacing cysteine with serine results in an inactive compound (CVIM, IC_{50} = 0.25 μM; SVIM, IC_{50} > 100 μM[25]). We speculated that if the thiol were replaced with a hydroxyl group of similar pKa, perhaps activity would be retained. Since phenols have pKa's close to those of thiols (pKa ~ 10), they seemed prime candidates as cysteine substitutes. Attachment of the phenol via a benzyl amine linkage (as opposed to a benzamide linkage) removes one peptide bond and should increase stability towards proteases. Thus, we prepared a series of CVFM analogs in which the cysteine was replaced by a hydroxybenzyl or thiolbenzyl unit. These compounds were tested as FTase inhibitors. The concentration to inhibit H-ras farnesylation by 50% (IC_{50}) is shown in Table 1.[27,37] None of the compounds in Table 1 were active against GGTase I (IC_{50} > 30 μM, data not shown).

The compounds used in this study were prepared by reductive amination of a salicylaldehyde derivative[38] with a VFM ester (typically a *p*-nitrobenzyl ester) as outlined in Scheme 1. Although an excess of the aldehyde was employed, only in the case of 2-formyl-3-hydroxypyridine[39] was a significant amount of over-alkylation observed (i.e., **19**). It was not necessary to protect the phenolic hydroxyl group during reductive amination, but thiophenol derivatives were protected. The requisite 2-mercaptobenzaldehydes were prepared by nucleophilic substitution of 2-fluorobenzaldehyde or 2-nitrobenzaldehyde with a thiol.[40]

Scheme 1



2-5 equiv.

In comparing compounds **1-4**, it is clear that the presence of a phenolic hydroxyl group *ortho* to the methylene linker improves activity against FTase approximately 10-fold over the simple benzyl derivative (**1**), while a hydroxyl in the *meta* or *para* position does not improve activity significantly. Naphthyl derivative **5** was 2-3 times less active than its phenyl counterpart (**2**). It seems unlikely that the observed preference of *ortho* vs. *meta* and *para* is due solely to a difference in pKa, since this difference would be expected to be small. Also no clear trend is seen with regard to the effect of electron-withdrawing vs. electron-donating substitution on the phenol ring. For example, the *p*-trifluoromethyl (**12**) (electron-withdrawing) and *p*-methoxy (**14**) (electron-donating) compounds differ in activity only by a factor of two. Compound **17**, however, which contains an electron-withdrawing (but bulky) ester group (*p*-$CO_2C_3H_7$), is completely inactive. The most active compounds contain a chloro or bromo substituent *para* to the hydroxyl on the phenol ring (**8**, **9**, **11**). Whether this improvement is due to an inductive effect on the hydroxyl (i.e., lowering the pKa) or to some direct interaction of the halogen atom with the enzyme is unclear; though since *ortho*-fluoro compound **6** is five times less active than **8** or **9**, the latter is implied. It appears that FTase activity is influenced by steric and electronic effects of substituents on the phenol.

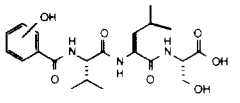
The hydroxypyridine derivative (**18**), which places a nitrogen nominally in the position of the amine of cysteine, was less active than the parent benzyl derivative (**1**). Interestingly, the methoxyethoxymethyl (MEM) ether (**9**) of chloro derivative **8** was almost as active as the free phenol. However, this result does not appear to be general since in the case of trifluoromethyl compound **12**, the MEM protected derivative (**13**) was 8-fold *less* active. One bisubstrate-type inhibitor[41] (**22**) was prepared (incorporating both farnesyl and CVFM), but was 30-fold less active than compound **2**.

Thiophenol replacements were briefly examined. It is interesting that the *ortho*-thiophenol derivative (**20**) is no more active than a simple benzyl derivative (**1**). This could be explained by a difference in pKa or bonding geometry in a thiophenol vs. a phenol. Thiophenols are approximately 2 pKa units more acidic than the corresponding phenols, the C–S bond is longer (by ca. 0.4 Å), and the C–S–H angle is more acute.[42] Not surprisingly, compounds in which the thiol is blocked by a bulky protecting group (e.g., diphenylmethyl (**21**) or *t*-butyl (not shown)) are completely inactive (IC_{50} > 100 μM). Since our aim was to find a non-thiol-containing replacement for cysteine, these thiophenol derivatives were not further investigated.

Also of note is compound **10**, in which one additional carbonyl has been removed (from **8**). Unlike in the case of CVFM, in which removal of the two N-terminal amide carbonyls is beneficial to inhibitory activity,[27] in this case reduction of this carbonyl results in a moderate decrease in activity. Thus, it appears that not all beneficial modifications to CVFM can be extrapolated to this phenolic series.

In the course of this work, Patel, *et al.*, reported a related series of compounds based on CVLS in which a hydroxybenzoic acid replaces cysteine (Fig. 2).[43] In contrast to the results presented herein, the *meta*-substituted derivative is the most active in Patel's series (IC_{50}'s: *ortho*, >360 μM; *meta*, 29 μM; *para*, >360 μM; CVLS, 1 μM). This difference in isomer selectivity between **2** - **4** and the compounds in Figure 2 is likely due to the amine vs. amide linkage connecting the phenolic portion to the tripeptide. Relative to the *amine* linkage present in the compounds in Table 1, an *amide* linkage would increase the acidity of the phenolic hydroxyl, reduce the flexibility of the N-terminus portion of the molecule, and, in the *ortho* isomer, likely tie up the hydroxyl proton in a hydrogen bond with the amide carbonyl. Another difference between these two series is that CVLS is a substrate inhibitor of FTase, while CVFM is a nonsubstrate inhibitor;[25,36] hence, since the A_2X portion of these CA_1A_2X tetrapeptides influences the thiol reactivity of cysteine, it could also affect the phenol portion of a peptidomimetic. Any or all of these effects could be responsible for the difference in selectivity between these two series of FTase inhibitors.

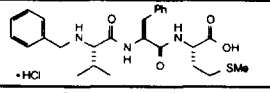
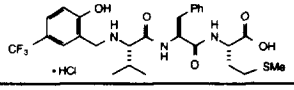
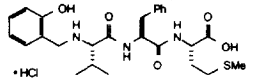
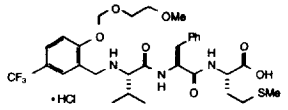
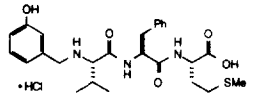
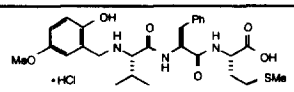
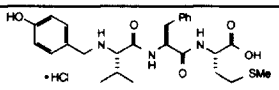
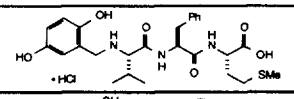
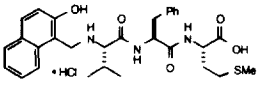
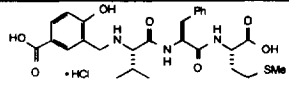
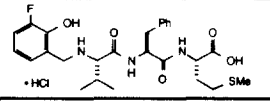
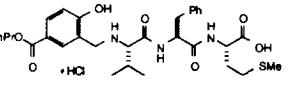
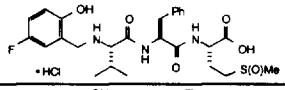
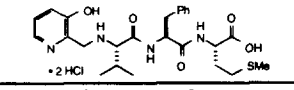
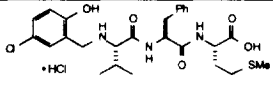
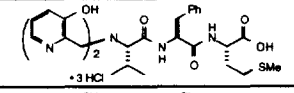
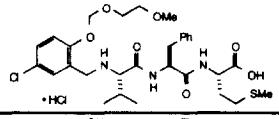
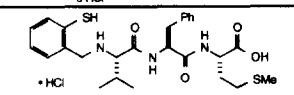
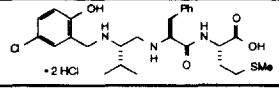
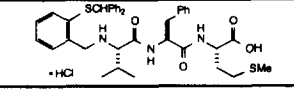
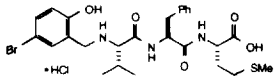
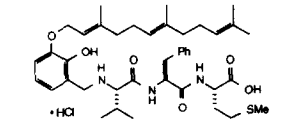
Figure 2. Analogs of CVLS



(Patel, *et al.*[43])

In conclusion, we have prepared a series of FTase inhibitors in which a phenolic benzyl group replaces cysteine in the tetrapeptide CVFM. The most potent compound is only eight times less active than CVFM, demonstrating that CVFM peptidomimetic inhibitors of FTase need not contain a thiol moiety to retain potency. This information is being used in the search for compounds that ultimately may be used to treat human cancers.

Table 1. Farnesyl Transferase Inhibitory Activity of Phenolic CVFM Analogs

	Compound	IC_{50} [μM] FTase		Compound	IC_{50} [μM] FTase
1		21	12		2.1
2		2.4	13		16
3		13	14		3.8
4		17	15		24
5		6.3	16		8.5-27.0
6		2.5	17		~ 100
7		1.6	18		30
8		0.50	19		30
9		0.76	20		22
10		2.3	21		~100
11		0.45	22		70

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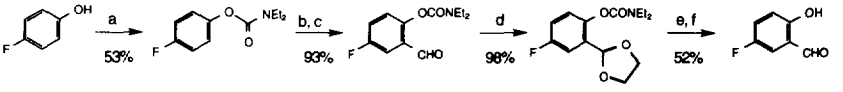
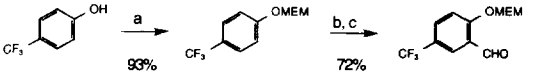
38. Benzaldehyde, 5-bromosalicylaldehyde, 5-chlorosalicylaldehyde, 2-fluorobenzaldehyde, 4-fluorophenol, 3-fluorosalicylaldehyde, 2,5-dihydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 3-hydroxy-2-(hydroxymethyl)pyridine hydrochloride, 2-hydroxy-5-methoxybenzaldehyde, 2-hydroxy-1-naphthaldehyde, and salicylaldehyde were purchased from Aldrich Chemical Co., Inc., 4-hydroxybenzotrifluoride from Lancaster Synthesis, Inc., and 3-formyl-4-hydroxybenzoic acid from Pfaltz & Bauer, Inc., and were used as received. *n*-Propyl 3-formyl-4-hydroxybenzoate was prepared from the acid by refluxing in 1-propanol with catalytic H_2SO_4.

5-Fluorosalicylaldehyde was prepared from 4-fluorophenol: (a) Et_2NCOCl, Et_3N, CH_2Cl_2; (b) *sec*-BuLi/TMEDA, THF -78 °C; (c) DMF; (d) $HOCH_2CH_2OH$, cat. pTsOH, benzene, reflux; (e) 4N NaOH, ethylene glycol, 100 °C, 7 h; (f) 1N HCl, CH_3CN, rt.



5-Trifluoromethylsalicylaldehyde derivatives were prepared from the MEM protected salicylaldehyde, which was prepared from 4-hydroxybenzotrifluoride: (a) MEMCl, DIEA, CH_2Cl_2; (b) *sec*-BuLi/TMEDA, THF, -78 °C; (c) DMF. The MEM group was cleaved from the final product (e.g., **13**) using $ZnBr_2$ in AcOH/TFA (1:100) at rt.



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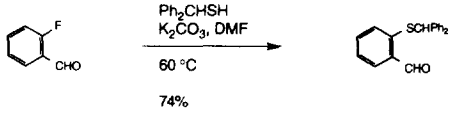

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